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An updated view on the differentiation of stem cells into endothelial cells

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Endothelial cells form the internal barrier between circulating blood and the vessel wall. They regulate arterial activity and mediate pathological reactions to vascular injuries such as atherosclerosis and balloon angioplasty. The development and differentiation of endothelial cells is a complex and coordinated process involving multiple levels of signaling and transcriptional and post-transcriptional regulation. Elucidating the mechanism of endothelial differentiation will not only enhance our understanding of vascular disease pathogenesis, but also facilitate our ability to produce vessels cells from pluripotent stem cells for regeneration purposes. In this review, we discuss the current understanding of how stem cells differentiate into endothelial cells at the level of signaling, transcription and microRNA regulation.

stem cells, endothelial cells, differentiation, signaling, transcription, microRNA

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The vascular system is fundamental for embryonic development and adult life because it is essential for the delivery of oxygen and nutrients to cells throughout the body. Endothelial cells (ECs) line the internal surface of the entire vascular system and form the barrier between circulating blood and the rest of the vessel wall. They serve to prevent thrombosis and to regulate arterial activity through synthesis and release of numerous vasoactive molecules. The endothelium is thus considered as a dynamic and heterogeneous organ with secretory, metabolic, synthetic and immunological functions [1]. In mammals, two distinct but related processes are involved in the establishment of blood vessels [2]: First, *de novo* vasculogenesis generates a primitive network of vasculature through the formation of ECs from endothelial precursor cells called angioblasts. Second, angiogenesis takes place with vessel expansion and further EC sprouting, branching and intussusception. Further specialization of the endothelial cells into arteries, veins, capillaries and lymphatic vessels ensures the proper functioning of the vasculature.

Differentiation of endothelial cells from their precursors involves co-operative interaction among many different signaling molecules and transcription factors (for reviews, see [3,4]). Understanding the molecular mechanism of EC differentiation will greatly benefit regenerative methods for treating certain vascular diseases, such as atherosclerosis, aortic dissections and aneurysms. Various methods have been applied to generate endothelial cells from pluripotent stem cells, which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), but often with limited efficiency. Thus, new techniques and refined protocols are needed to produce sufficient numbers of a desired cell type for tissue engineering and regenerative medicine. Over recent years, there has been great progress in our under-

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standing of how molecules participate in endothelial lineage commitment. In this review, we discuss the signaling pathways and transcriptional regulation of endothelial differentiation and also the involvement of microRNAs during this process.

1 Signaling pathways involved in the regulation of endothelial differentiation

Establishment of endothelial cells occurs both extraembryonically and intraembryonically during mammalian embryonic development. In the yolk sac, mesodermal precursors of hematopoietic and endothelial lineages differentiate into a cluster of cells called blood islands, where cells in the inner part of the island give rise to hematopoietic cells and cells in the outer part differentiate into endothelial cells [3]. The subsequent coalescence of blood islands and the formation of the lumina lead to a primitive vascular plexus. Within the embryo, endothelial precursor cells called angioblasts migrate and differentiate to form the primordial aorta. Simultaneous migration of angioblasts from presomitic cranial mesoderm forms the endocardial tube in the pericardial area [4].

Several signaling pathways, including those for hedgehog (Hh), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), bone morphogenetic protein (BMP), Notch and Wnt, are required for induction of endothelial and hematopoietic lineages within the endoderm [5]. Although the current model of signaling hierarchy controlling endothelial cell development is largely derived from studies of mouse, endothelial differentiation in different mammalian species seems to adopt a similar signaling program [6,7].

1.1 Hedgehog signaling

Signaling by the Hedgehog family of secreted proteins plays a key role in embryonic patterning and morphogenesis [8] and is essential for vertebrate blood vessel formation [9]. The Hedgehog family consists of three members: sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH). IHH, a primitive endoderm-secreted protein, is sufficient to induce formation of endothelial and hematopoietic cells in the mouse embryo with the endoderm removed [10]. SHH, a more widely expressed protein during embryogenesis and which shares a redundant role with IHH [11], was also shown to be both necessary and sufficient for endothelial tube formation from angioblasts in avian embryos [12]. Zebrafish embryos mutant in the SHH pathway display a defect in vascularization and a loss of arterial differentiation, and overexpression of SHH leads to the formation of lumenized ectopic vessels [13]. Embryoid bodies formed from mouse embryonic stem cells deficient in either *Ihh* or a receptor component of the Hh pathway, *Smoothened* (*Smo*), are unable to form blood islands and express no endothelial cell markers [14]. However, $Ihh^{-/-}$ embryo yolk sacs can form blood vessels, although the vessels are fewer in number and are smaller [14], indicating that Hh signaling may play an inductive role in directing mesoderm differentiation but not a direct role in endothelial cell differentiation.

1.2 Bone morphogenetic protein signaling

Bone morphogenetic proteins function downstream of Hedgehog signaling [6,7] and are also critical for regulating embryonic vascular development. First identified in extracts from bone matrix and with a role of promoting bone formation, BMPs are now known as essential regulators of the development of nearly all vertebrate organs systems, including the embryonic vasculature. *Bmp4* knockout mice are embryonic lethal due to failed generation of mesoderm [15]. Embryos deficient for downstream BMP signaling molecule *Smad5* lack organized yolk sac vasculature [16]. BMP4 initiates efficient induction of mesoderm in human embryonic stem cells [17,18] and can promote formation of $Flk\text{-}1^+Scl^+$ hematopoietic and endothelial progenitors [19], $CD34^+$ progenitors [20], $CD34^+CD31^+$ vascular progenitors [21], $CD144^+KDR^+$ endothelial progenitors [22] and hemogenic endothelium from murine ESCs [23].

The VEGF receptor, Flk-1, is now recognized as a target of BMP signaling. BMP4, via SMAD1/5 signaling, induces differentiation of ESCs into $Flk\text{-}1^+$ cells in serum-free conditions [19]. Recent studies have identified several transcription factors that mediate BMP4-induced EC differentiation. CXXC5, a nuclear transcription factor containing a CXXC-type zinc-finger domain, is induced during endothelial differentiation from mouse ESCs and mediates transcriptional activation of *Flk-1* via BMP4-Smad signaling [24]. *Cxxc5* morpholino-injected zebrafish display defects in caudal vein plexus formation, and $Cxxc5^{-/-}$ mice with subcutaneously injected matrigel plugs show suppressed BMP4-induced angiogenesis, suggesting that *Cxxc5* is required for vessel formation. ER71/ETV2, a member of the ETS (E26 transformation-specific or E-twenty-six) transcription factor family, promotes BMP4-mediated $Flk\text{-}1^+$ cell development and overexpression can rescue the formation of $Flk\text{-}1^+$ mesoderm blocked by BMP [25]. Another study showed that *Gata2*, one of the blast colony-forming cell (BL-CFC)-enriched transcripts, was a direct target of BMP4 and was able to promote generation of $Flk\text{-}1^+$ mesoderm and $Flk\text{-}1^+Scl^+$ hemangioblasts [26].

1.3 WNT signaling

The WNT family of proteins are secreted glycoproteins that control fundamental aspects of development, including embryonic patterning, cell fate specification, survival, and overall organogenesis [27]. There are three main WNT sig-

naling pathways: the canonical WNT/β-catenin pathway, the WNT/Ca^{2+} pathway, and the planar cell polarity (PCP) pathway. Canonical WNT signaling is the most extensively studied and plays a critical role in vascular development. Deletion of β-catenin in developing endothelial cells leads to embryonic death and loss of EC integrity [28]. The canonical WNT pathway is required for primitive streak formation [29,30] and initial formation of mesoderm from the pluripotent epiblast or mouse ESCs. Further differentiation of ESCs into hematoendothelial cells still requires canonical WNT signaling [31]. Inhibition of WNT leads to decreased generation of Flk-1^+ cells [32], whereas activation of the WNT signal is able to expand Flk-1^+ vascular progenitor populations [33]. A definite role of WNT signaling in directing differentiation of embryonic stem cells into the endothelial lineage has been identified with *Wnt5a* deficient murine ESCs [34]. *Wnt5a*$^{-/-}$ mouse ESCs exhibited a defect in endothelial differentiation, which could be rescued by adding recombinant WNT5a protein. In addition, both the canonical β-catenin and the PKCα-mediated non-canonical signaling pathways are required for the differentiation of mESCs to ECs induced by Wnt5a [34].

1.4 VEGF signaling

VEGF signaling is the key regulatory pathway controlling vascular development and diverse endothelial cell functions, including cell survival, proliferation, migration and vascular permeability [35]. Among the five members of the VEGF family, which includes VEGFA, VEGFB, VEGFC, VEGFD and VEGFE, VEGFA is the most functionally significant and best characterized. VEGFA signals via its main receptors, VEGFR1 (fms-related tyrosine kinase-1, Flt-1) and VEGFR2 (fetal liver kinase-1, Flk-1), and can also bind to semaphorin receptors, neuropilin (NRP)1 and NRP2. The impact of VEGFA on endothelial cell development and function is mainly achieved through binding to its tyrosine kinase receptor VEGFR2 with activation of downstream effectors, including PKC, PLCγ, MAPK and the PI3K/Akt/PKB cascade [36]. In contrast to VEGFR2, VEGFR1 functions as a decoy receptor with stronger affinity for VEGFA but less tyrosine kinase activity, thus negatively regulating VEGF signaling.

Heterozygous mutants of *Vegfa* and homozygous mutants of *Vegfr2* are embryonic lethal at embryonic day (E) 11–12 and E8.5–9.5, respectively, due to defective blood-island formation and impaired vascular development [37,38]. However, *Flk-1*$^{-/-}$ mouse embryonic stem cells are able to differentiate into hematopoietic and endothelial cells, although their subsequent migration and expansion requires a Flk-1-mediated signal, suggesting that VEGF signaling is dispensable for endothelial cell differentiation but may instead regulate EC survival [39,40]. Similarly, VEGF does not promote endothelial cell differentiation from human embryonic stem cells [7]. For VEGFR2$^+$ mesodermal vascular progenitor cells, phosphorylation of VEGFR2 Y1175 and subsequent activation of the VEGFR2-PLCγ1 axis and downstream Ras signaling is vital for endothelial lineage specification [41,42].

1.5 Notch signaling

Notch signaling is of vital importance in vascular development, and participates in processes such as vascular smooth muscle cell differentiation, arterial-venous cell fate specification, EC proliferation and angiogenic sprouting [43]. However, the role of Notch signaling in endothelial differentiation has not been defined. In growing vascular sprouts, VEGF induced the activation of Delta-like/Notch signaling, which suppresses tip cell fate in neighboring cells. One recent study expanded the prevailing model, showing that in endothelial progenitors (EPs), activation of the Notch pathway via VEGF-VEGFR2 signaling feeds back to inhibit *Vegfr2* transcription and thereby limits EP maintenance and amplification [44]. Thus, VEGFA stimulation with Notch inhibition enhances EP formation and amplification, and blocks conversion of EPs to mature ECs. Moreover, Notch signaling regulates further EC specification by maintaining an arterial fate and inhibiting venous or lymphatic lineages [45].

2 Transcriptional and epigenetic regulation of endothelial differentiation

The specification of endothelial lineages requires a coordinated regulation of signaling molecules and transcriptional factors as well as epigenetic modifiers to direct endothelial-specific gene expression. The past decade has witnessed tremendous progress in dissecting the function of these transcription factors; however, the transcriptional regulatory network governing endothelial differentiation remains incompletely understood. Here we summarize several of the transcription factors and epigenetic mechanisms that are involved.

2.1 ETS transcription factors

E-twenty six (ETS) proteins are a group of DNA-binding transcription factors that are critical for endothelial development. ETS family members share a highly conserved ETS domain that binds to a core 5′-GGA(A/T)-3′ sequence in target genes. To date, 26 mouse and 27 human ETS proteins have been identified [46], and 19 of them are expressed in human endothelial cells. All identified endothelial enhancers and promoters contain multiple ETS binding motifs [3], indicating that ETS factors may control most endothelial-related genes. Among the ETS factors regulating endothelial development, such as Ets-1, Erg, Fli-1, and Etv2, Etv2 (also known as Er71, ETS-related 71) is by far

the best characterized.

During embryogenesis, *Etv2* expression is initially widely spread within the primitive streak mesoderm, and then becomes more limited to major vessels at E8.5–9.5, and finally disappears beyond E10.5–11.5 [25,47]. This dynamic change of *Etv2* expression reflects its role in early vascular development. In fact, Etv2 is a potent activator of several early endothelial genes, such as *Flk1*, *Tal1*, *Mef2c*, *Pecam* and *Tie2* [3]. *Etv2* knockout mice are embryonic lethal due to a complete lack of blood vessels and hematopoietic cells [25,48], and morpholino knockdown or mutation of *etsrp*, the *Etv2* ortholog in zebrafish, results in complete deficiency in endothelial development [49,50]. Etv2 activation is critical for driving Flk-1$^+$ mesoderm differentiation and specifying Flk-1$^+$ PDGFRα^- hematopoietic and endothelial cell lineages, while suppressing the Flk-1$^+$ PDGFRα^+ cardiac lineage [47,51,52], partly through inhibiting the WNT signaling pathway [53]. On the other hand, ensuring a tightly controlled *Etv2* function is critical for proper development of the vascular system. Transgenic mice with constitutive *Etv2* expression induced by *Tie2-Cre* display dilated yolk sac vessels accompanied with hemorrhaging and are eventually embryonic lethal [54]. In a recent study, transient *Etv2* expression with constitutive *Erg1* and *Fli1* co-expression could reprogram amniotic cells into durable endothelial cells [55]. In summary, Etv2 plays a central early role in endothelial specification and acts near the top of the transcriptional network governing endothelial cell development.

ETS factor Fli also acts at the top of the transcriptional network governing hematopoietic and endothelial development. Knockdown of *Fli* in *Xenopus* substantially inhibits hematopoietic and endothelial differentiation, and activation of *Fli* transactivates early hemangioblast genes through *Scl*, *Etsrp* and *Cloche* [56]. However, disruption of *Fli* in zebrafish fails to recapitulate the defects in *Xenopus*, and *Fli1*$^{-/-}$ mouse embryos do not display severe vascular defects but die of hemorrhage with loss of vascular integrity [57,58], indicating that *Fli* is dispensable for EC development in zebrafish and mouse, but is required for vasculature formation after endothelial establishment. As a matter of fact, *Fli* acts downstream of *Etv2* via a positive feed-forward regulatory loop during vasculogenesis [59].

Redundancy between different ETS factors may explain the absence of vascular defects in knockout mouse studies. Mice with nonfunctional *Erg*, an ETS factor highly homologous to *Fli*, displayed no overt vascular phenotype with only dilated vessels in the brain [60]. Subsequent studies showed Erg regulated angiogenesis and endothelial survival by inducing adhesion molecule VE-cadherin and modulating HDAC6 function [61,62].

2.2 Forkhead transcription factors

The Forkhead (Fox) transcription factors play essential roles in vascular biology and endothelial function, and members that are of major importance in endothelial development include the FoxC, FoxF, FoxH and FoxO families [63]. *Foxo*1-deficient mice are embryonic lethal due to deregulated vascular development, including malformation of the aorta and internal carotid artery branches [64,65]. While mice deficient in *Foxo3* did not show vascular defects, overexpression of Foxo1 and Foxo3 inhibited endothelial migration and tube formation, indicating a suppressive role of FOXO transcription factors in the regulation of vessel formation [66]. *Foxf* knockout mice showed defective mesodermal differentiation and disordered vascular patterning in the yolk sac [67]. *Foxc1*$^{-/-}$ and *Foxc2*$^{-/-}$ knockout mice are lethal by E9.5 with defective vascular remodeling and arteriovenous malformations [68].

FoxC proteins are important cofactors for ETS transcription factors, which together regulate endothelial gene expression [69]. Forkhead and ETS transcription factors can synergistically activate a composite *cis*-acting element called the FOX:ETS motif present in enhancers or promoters of a variety of endothelial genes such as *VE-cadherin*, *Pecam1*, *Tie2*, and *Scl* [69]. Coexpression of *foxC2* and *etv2* induces ectopic expression of vascular genes in *Xenopus* embryos, and simultaneous knockdown of the orthologous genes disrupts vascular development in zebrafish embryos [69]. In a recent study, *foxc1a/b* was found to be a direct upstream regulator of *etsrp* in zebrafish [70]. Combined knockdown of foxc1a/b led to a significant decrease in *etsrp* expression at early developmental stages, indicating that coordination of FoxC with ER71 is critical in mediating EC development [70].

2.3 GATA transcription factors

GATA transcription factors were initially thought to be required for hematopoietic development, because some early studies showed *Gata1*$^{-/-}$ and *Gata2*$^{-/-}$ mice to be defective in erythropoiesis and to display anemia [71,72]. Subsequent studies found GATA also played an important role in hemangioblast formation and endothelial development [26]. GATA2 can induce the generation of Flk-1$^+$ mesoderm, the Flk-1$^+$ Scl$^+$ hemangioblasts and endothelial cells [26]. In fact, GATA binding sites are present in several endothelial genes, such as *Flk1* and *VE-cadherin* [73,74], and GATA regulates the endothelial gene, *Endomucin*, through epigenetic modulation [75].

2.4 Epigenetic modifications

Epigenetic control of EC differentiation involves DNA methylation of endothelial lineage promoters and post-translational modification of histones. During differentiation, promoter regions of the genes involved in endothelial fate determination and homeostasis undergo intensive hypomethylation, allowing more efficient transcription [76–78].

Early studies showed inhibition of histone deacetylases (HDACs), enzymes that remove acetyl groups from lysine residues of histone proteins, blocked the endothelial differentiation of adult progenitor cells [79]. In contrast, laminar flow-induced activation of HDAC3 can promote EC differentiation [80]. Histone methylation with H3K27me marks an epigenetically silenced state, while H3K4me permits gene activation. By pharmacologically inhibiting histone methyltransferase to reduce repressive H3K27me, endothelial progenitor cells (EPCs) increased expression of endothelial nitric oxide synthase (eNOS), and the reduction of H3K27me was associated with an increase in the expression of the histone demethylase, *Jmjd3* [81]. Ablation of *Jmjd3* expectedly compromised mesoderm and subsequent endothelial differentiation, partly by silencing the mesodermal regulator *Brachyury* and reducing β-catenin recruitment [82]. On a practical level, targeting epigenetic repressive marks would yield more ECs differentiated from stem cells so as to enhance their function for bioengineering applications [83].

3 microRNAs and EC differentiation

microRNAs (miRNAs) are small, non-coding RNAs that play important regulatory roles in various aspects of development, homeostasis and disease by pairing to the mRNAs of protein-coding genes as negative and positive post-transcriptional regulators [84–86]. miRNAs are known to control the self-renewal and differentiation program of ES cells [87,88]. Numerous miRNAs have been shown to promote the differentiation of ESCs into various cell lineages, including cardiomyocytes [89], endothelial cells [90], smooth muscle cells [91] and skeletal muscle cells [92]. The switch from pluripotent to lineage-specific state by miRNAs involves repressing the self-renewal program by inhibiting core pluripotent factors and inducing the expression of lineage-specific gene products.

Global expression of miRNAs is essential for vascular development. Deletion of *dice*r in mouse led to embryonic death, due to defective blood vessel formation and maintenance [93,94]. In line with this observation, a mouse mutant with a hypomorphic *dicer1* resulted in female infertility caused by impaired growth of new capillary vessels in the corpus luteum [95]. Endothelial-selective ablation of *dicer* in mouse led to significant defects in postnatal angiogenesis in response to a variety of stimuli such as exogenous VEGF, ischemia, and wound healing [96]. Consistent with these observations, *in vitro* studies showed that knockdown of Dicer in human endothelial cells reduced capillary sprouting and tube formation by silencing several critical target miRNAs, such as lef-7f and mir-27b [97,98].

Roles of several miRNAs, including miR-126 [99,100], miR-221 [101], miR-132 [102], miR-218 [103,104], miR-23~27~24 clusters [105], miR-27a/b [106] and miR-92 [107], have been carefully examined in the regulation of vascular development, angiogenesis and endothelial functions through the fine-tuning of VEGF, Notch and Slit/Robo signaling pathways. Though many of the aforementioned miRNAs have been shown to regulate proper EC function, little is known about their role in EC differentiation. Some recent studies have identified several sets of miRNA that may participate during this process [90,108,109], although many of them have not been directly tested with respect to being able to direct EC differentiation. Here, we summarize only those miRNAs with a definite role in promoting EC differentiation [110] (Figure 1).

3.1 miR-21

As one of the first mammalian miRNAs to be identified, miR-21 has been extensively studied and was found to be deregulated in many pathological conditions, including cancer and organ fibrosis [111]. Apart from its classic role as a tumor suppressor, recent studies have highlighted miR-21 as an important mediator during pathogenesis of cardiovascular disease, including angiogenesis [112], myocardial infarction [113,114] and ischemia/reperfusion [115], and in advanced peripheral arterial disease [116]. A recent genetic study on deletion of the neuronal repressor REST (RE1-silencing transcription factor) in embryonic stem cells also indicated a role of miR-21 in regulating stem cell self-renewal and differentiation [117]. REST is expressed at high levels in mouse embryonic stem cells, and is able to suppress a set of miRNAs that suppress stem cell self-renewal and promote differentiation. Among them, miR-21 reduces the expression of pluripotent factors Oct4, Nanog, Sox2 and c-Myc and specifically inhibits the self-renewal of

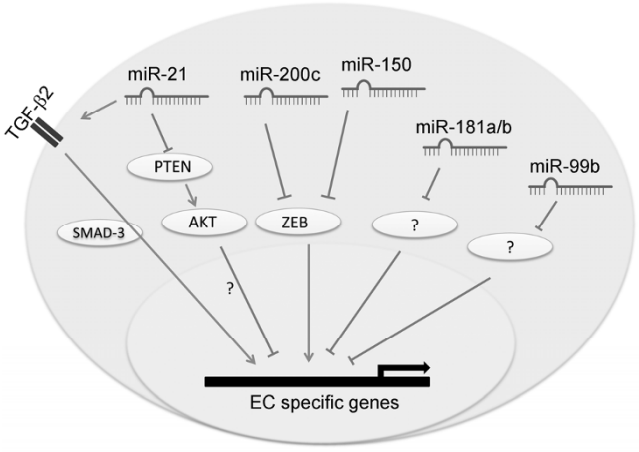

Figure 1 Roles of miRNAs in endothelial differentiation. miR-21 promotes endothelial differentiation through inhibition of the PTEN/Akt pathway. MiR-21 also increased TGF-β2 production and subsequent EC gene activation in a SMAD3-dependent manner. MiR200c and miR-150 induce endothelial differentiation by transcriptionally de-repressing ZEB1 expression. miR-181a/b and miR-99b mediate EC lineage specification through as yet unknown mechanisms.

mouse ES cells. Later studies showed that miR-21 is able to regulate differentiation of other cell lineages including monocyte-derived dendritic cells (MDDCs) [118], granulocytic cells [119] and adipogenic cells [120].

Our recent study on endothelial differentiation from induced pluripotent stem cells also indicated a role of miR-21 in regulating the TGF-β signaling pathway [121]. Overexpression of miR-21 in pre-differentiated iPSCs induced capillary formation *in vitro* and *in vivo*, and increased levels of TGF-β2 mRNA and secreted protein. TGF-β2, in turn, promotes iPSCs differentiation towards the endothelial lineage through a SMAD3-dependent pathway. During endothelial cell differentiation, miR-21 targets the PTEN/Akt pathway and PTEN knockdown is required for miR-21 mediated upregulation of EC markers in differentiated iPSCs.

3.2 miRNA-200 family

The miR-200 family consists of five members: miR-200a, miR-200b, miR-200c, miR-141 and miR-429. These five miRNAs are located in two separate chromosomal clusters with the miR-200b-200a-429 cluster at chromosomal location 1p36 and miR-200c-141 cluster at 12p13. By targeting E-box-binding homeobox (ZEB) transcription factors, the miR-200 family negatively regulate epithelial-to-mesenchymal transition (EMT) [122], which is crucial for embryogenesis and certain pathophysiological conditions, such as wound healing and carcinoma progression [123]. Expression of miR-200 is reduced in normal mammary stem cells, breast cancer stem cells (CSC) and embryonic carcinoma cell lines [124] and is up-regulated during ESC differentiation [125,126]. Overexpression of miR-200 suppresses expression of stem cell factors Bmi1 and Sox2 in CSCs and mouse ESCs, and induces differentiation [124,126].

The ZEB-miR-200 interplay is also involved in endothelial differentiation of human ESCs [90]. ZEB1 transcriptionally repress EC-specific gene expression during EC differentiation. miR-200c de-represses this effect by inhibiting ZEB1 transcription, and thus promotes endothelial lineage differentiation. In a Matrigel-CD146$^+$ EC-committing cell mixture implanted in mice, blocking ZEB1 signaling can rescue the inhibitory effect of miR-200c inhibition on *in vivo* vasculogenesis [90]. Interestingly, miR-200c promotes mesoderm specification while repressing neuroectodermal differentiation from ESCs [127], suggesting that miR-200c may play a cell-autonomous role in mediating EC differentiation from stem cells.

3.3 miRNA-150

miR-150 is classically involved in hematopoiesis and regulates cell differentiation in both lymphoid and myeloid lineages [128]. miR-150 is considered to be a tumor suppressive gatekeeper in leukemogenesis [129], and its aberrant expression is critical for pathogenesis in a variety of hematopoietic malignancies. The impact of miR-150 on endothelial cell differentiation seems similar to that of miR-200c, in that miR-150 also promotes endothelial lineage specification by transcriptionally repressing ZEB1 expression [90]. However, mice deficient in miR-150 are viable, fertile, and morphologically normal [130], indicating that the signaling networks in which miR-150 functions may not be essential for embryonic vasculogenesis, although such discrepancies could be attributed to the compensatory effects of other molecules in the knockout mice.

3.4 miRNA-181

The miR-181 family is composed of six members: miR-181a1/2, miR-181b1/2, miR-181c, and miR-181d. They are expressed in a number of tissues, including muscle, eye, brain, lung and the hematopoietic compartment [131]. miR-181 family members play critical roles in controlling cardiovascular inflammation by regulating critical signaling pathways, such as the NF-κB pathway and molecules relevant to endothelial cell activation [132] and immune cell homoeostasis [133].

In a recent study employing miRNA microarray analysis during defined stages of EC differentiation from human ESCs, miR-181a and -181b were identified as increasing in a time-dependent manner to peak in mature hESC-ECs [109]. Overexpression of miR-181a and -181b enhanced levels of EC-specific genes, increased nitric oxide production, and improved hES-EC-induced therapeutic neovascularization in an *in vivo* model of peripheral ischemia. Knockdown of miR-181a and -181b significantly reduced vascular endothelial markers and nitric oxide production but had no effect on the cell population expressing endothelial marker proteins [109], suggesting that miR-181a and -181b may not be essential for EC lineage specification. This was confirmed in another knockout study of mice deficient in miR-181 that showed no obvious gross phenotypic abnormalities in terms of growth, development, or survival [134].

3.5 miR-99 family

The miRNA-99 family consists of miR-99a, -99b, and -100. They predominantly act as tumor suppressors by inducing cell cycle arrest [135] and inhibiting cell proliferation [136]. The miR-99 family also modulates injury responses, such as post-radiation DNA damage [137] and dermal wound healing [138]. miR-99b was co-identified with miR-181 in the study mentioned above as promoting EC differentiation from ESCs [109]. Like miR-181, augmentation of miR-99b induced EC-specific marker expression and nitric oxide generation, but its knockdown did not impact endothelial differentiation. This indicates that although capable of promoting EC differentiation from pluripotent ESCs, miR-99 is dispensable for EC differentiation.

3.6 Other miRNAs

A series of new miRNAs regulating endothelial differentiation have been identified by Yoo et al. [139–141], but none of them have been functionally characterized. Among them, miR-5739 and miR-6087 modulate the expression of endoglin [139,140], miR-6078 targets the E-cadherin (Cdh) gene [140], while miR-7641 suppresses expression of CXCL1 [141].

4 Conclusion

Recent years have witnessed progress in deciphering the molecular mechanism of endothelial differentiation; however, a comprehensive understanding of the exact differentiation program is still far from complete. Tissue engineering and stem cell therapy has important clinical implications for treating vascular diseases such as atherosclerosis and artery dissections; therefore, a better understanding of the EC differentiation program will greatly facilitate our ability to generate vessel cells from pluripotent stem cells. With the invention and maturation of iPS techniques, more specifically tailored cells that can be injected intravascularly will be made for producing bio-compatible vessels. As more details emerge of how endothelial cells are differentiated from stem cells, harnessing these differentiation mechanisms to enable the generation of endothelial cells from pluripotent stem cells will undoubtedly have future therapeutic implications of clinical value.

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